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Structural and Functional Properties of Complement-activating Protein M161Ag, a *Mycoplasma fermentans* Gene Product That Induces Cytokine Production by Human Monocytes*

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From the [‡] Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537, the [§] Department of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060, and [¶] PRESTO, JST, Tokyo 105, Japan

ABSTRACT

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Human malignant cells are targeted by homologous complement C3b if they express M161Ag, a 43-kDa protein with C3-activating property. cDNA of M161Ag cloned from human leukemia cell lines predicted M161Ag as a novel secretory protein comprised of 428 amino acids including 5 amino acids encoded by TGA codons (Matsumoto M., Takeda, J., Inoue, N., Hara, T., Hatanaka, M., Takahashi, K., Nagasawa, S., Akedo, H., and Seya, T. (1997) *Nat. Med.* 3, 1266-1270), although the origin of this gene was obscure. Here we clarified this point through genomic and biochemical analysis: 1) 5'-UT and genomic sequences represented the prokaryote promoter and ribosomal binding site; 2) the TGA codons in M161Ag cDNA were translated not into selenocysteines but into tryptophans; 3) M161Ag anchored onto the membrane secondary to its N-terminal palmitoylation like prokaryote lipoproteins; 4) genomic and cDNA clones of M161Ag were highly homologous to *Mycoplasma fermentans* gene encoding P48, a monocytic differentiation/activation factor, recently released in the data base, although the resultant proteins were different in the amino acid sequences. Additionally, purified soluble M161Ag efficiently provoked IL-1β, tumor necrosis factor α, and IL-6 like P48, and further IL-10 and IL-12 in human peripheral blood monocytes. Thus, M161Ag originates from *M. fermentans*, and latently infected *M. fermentans* allows human cells to produce M161Ag. The liberated protein serves as a potent modulator of innate and cellular immune responses via

its complement-activating and cytokine-producing activities.

INTRODUCTION

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Selective tumor destruction has long been desired for tumor immunity. Recently we discovered a membrane-associated novel gene product expressed on some malignant human cells/cell lines, but not on normal cells, in close conjunction with apoptotic stimuli such as Fas or x-irradiation. This protein, with a molecular mass of 43 kDa and named M161Ag (1-3), activates homologous complement (C).[1] The C-opsonized tumor cells are rapidly cleared, presumably through the expressed M161Ag.

Its cloned cDNA, however, suggested that M161Ag was a secretory protein and suprisingly contained 5 amino acids encoded by TGA codons (3). A possible C-activation characteristic of M161Ag is that once secreted from the tumor cells, it activates homologous C via the alternative pathway on the cell membrane, thereby allowing for homologous C3 targeting (3). Thus, M161Ag appeared to have unique structural, functional and expression profiles. Yet, information about the relevant amino acids encoded by TGA codons, genomic organization, the type of protein anchoring onto membranes, and the regulatory mechanism of protein expression remain to be settled.

We previously thought that the M161Ag gene was of human origin because M161Ag was expressed on malignant human cells from patients. However, a similar DNA sequence of non-human origin, named P48, was released after the submission of the amino acid sequence of M161Ag (3). The P48 protein was first described as a novel human cytokine inducing the production of IL-1β, TNF-α, and IL-6 in human monocytes and then corrected into a product of *Mycoplasma fermentans*, a parasitic prokaryote (5-8). Irrespective of the high homology between these two genes, there are significant differences in the primary structures as well as functional profiles of these gene products. Meanwhile, several papers have been published suggesting that leukemia (9) and AIDS (10) are frequently associated with parasitic *M. fermentans* and its gene products.

Here we demonstrate that M161Ag is a lipoprotein derived from *M. fermentans*. Furthermore, we found that M161Ag is a potent biological response modifier that provokes IL-10 and IL-12 in addition to inflammatory cytokines in human monocytes.

MATERIALS AND METHODS

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Reagents, Cell Lines, and Cells-- Monoclonal antibodies (mAbs) against M161Ag (M161 (2), MK53 (11)) were produced in our laboratory and purified on DEAE-Sephacel or Protein G-Sepharose (Pharmacia, Uppsala, Sweden). Restriction enzymes were purchased from Takara (Kusatsu, Japan). ELISA kits for TNF-α, IL-1β and IL-6 were purchased from Perseptive Biosystems (Framingham, MA), and those for IL-10 and IL-12 were from Endogen (Woburn, MA).

Human leukemia cell lines (P39, THP-1, HL60, CEM, and MT2), fibrosarcoma (HT1008), and lung

fibroblasts (WI38) were donated by the Japanese Cancer Research Resources Bank (JCRB). K562 and U937 were gifts from Dr. J. P. Atkinson (Washington University, St. Louis, MO), Jurkat was from Dr. S. Nagasawa (Hokkaido University), and primary cultures of human fibroblasts were from Dr. J. Takeda (Osaka University). These cell lines were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% FCS (CSL Limited, Victoria, Australia) in the presence of antibiotics.

Peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN) were isolated from venous blood of normal volunteers by methylcellulose and Ficoll-Hypaque sedimentation methods. Monocytes were prepared from PBMC as described (12).

Southern Blotting Analysis-- Human genomic DNA was isolated from peripheral blood leukocytes, primary cultures of human fibroblasts, various leukemia cell lines, and spleen using an Iso Quick nucleic acid extraction kit (ORCA Research Inc., Bothell, WA) or QIAamp tissue kit (Qiagen Inc., Chatsworth, CA) according to the manufacturers' directions. For each digest, 10 μg of DNA was digested with *Eco*RV, *Kpn*I, *Bam*HI, or *Hae*III and then separated by electrophoresis on a 0.7% agarose gel. The DNA was transferred onto Hybond N^+ nylon membranes (Amersham, Buckinghamshire, UK) and immobilized using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). The blots were prehybridized for 30 min, hybridized for 2 h at 65 °C in rapid hybridization buffer (Amersham) with ^{32}P-labeled full-length M161Ag cDNA, and washed at high stringency (65 °C, 0.2 × SSC, 0.1% SDS). The membranes were exposed to Kodak Hyperfilm™ MP for 24 h at −70 °C.

Cloning of Genomic M161Ag Gene-- Genomic DNA from WI38 cells (50 μg) was digested with *Hae*III at 37 °C overnight and run on a 0.7% agarose gel. An ~3-kb fragment was recovered from the gel using a QIAEX II kit (Qiagen) and ligated into *Eco*RV-digested pBluescript II KS^+ vector (Stratagene) with T4 ligase. The ligated DNA was transformed into competent MC1061 cells by electroporation using a Gene-Pulser (Bio-Rad). The transformants consisting of 1.21×10^4 colonies were divided into 20 tubes (600 colonies/tube), cultured at 37 °C overnight, and then subjected to PCR screening for the M161Ag gene using specific primers, 5'-TTGAGTCCTATTGCTGCTATTC-3' and 5'-CACCAAATGATGCAACAACTCT-3'. Colonies (5×10^3) from one positive tube were screened for the M161Ag gene by colony hybridization using a ^{32}P-labeled full-length M161Ag cDNA. Positive clones were subjected to sequence analysis.

DNA Sequencing and Computer Analysis-- DNAs were sequenced on both strands using a dideoxy terminator cycle sequencing kit (Applied Biosystems). Homology search was performed on NCBI, and nucleotide/protein analysis was performed with Gene Works, GENETYX, Clustal W in a Macintosh 7200.

RT-PCR and PCR-- Poly$(A)^+$ RNA from M161Ag-positive cell lines were reverse-transcribed using a random primer with RNase H-free reverse transcriptase (Superscript, Life Technologies, Inc.). The full-length M161Ag was amplified using forward 5'-AAGGAGATTATATGAAAAAGTC-3' and reverse 5'-AAGTGTACTTCTCTAGTCAATC-3' primers with exTaq (Takara). The thermocycle conditions were 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 2 min for denaturation, annealing and extension, respectively. PCR products were cloned into the pCR™II vector (Invitrogen)

and sequenced. PCR reactions with *Mycoplasma*-specific primers were run according to the manufacturer's directions (Takara) using 1 µg DNA template. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

Protein Isolation-- Purified MK53 was coupled to CNBr-activated Sepharose 4B. M161Ag was purified from P39(+) cell lysates (5×10^9) using column chromatography as described previously with slight modifications (2). After the Q-Sepharose and chromatofocusing columns, fractions containing M161Ag were pooled, and the pH was adjusted to 7.4 and applied to a MK53-Sepharose equilibrated with 10 mM Tris-HCl, 0.14 M NaCl, 0.02% Nonidet P-40, 0.5 mM PMSF, pH 7.4. The column was sequentially washed with starting buffer containing 0.5 M NaCl and then starting buffer without Nonidet P-40. M161Ag was eluted with 0.1 M triethylamine, 0.5 mM PMSF. Prior to amino acid analysis, the eluate was further purified by high performance liquid chromatography (HPLC) using a Phenyl-5PWRP column (4.6 × 75 mm, Tosoh Corp., Tokyo) and a HPLC Cosmosil $5C_4$-AR-300 column (4.6 × 150 mm, Nakalai tesque, Kyoto). In each step, protein elution was checked by immunoblotting. The final sample gave a single band on 12.5% SDS-PAGE and silver staining.

Secreted M161Ag was purified from conditioned media (CM) of P39(+) cells in the absence of Nonidet P-40. Five liters of CM were concentrated by 50% ammonium sulfate precipitation and dialyzed against 20 mM PBS, 0.5 mM PMSF, pH 6.0, overnight at 4 °C. The sample was applied to an S-Sepharose column equilibrated with the same buffer and eluted with 1 M NaCl in the starting buffer. M161Ag-positive fractions (checked by immunoblotting) were pooled and dialyzed against PBS, 0.5 mM PMSF, pH 7.4. The soluble M161Ag was further purified by the MK53-Sepharose as described in the purification of the membrane-bound form. The buffer was exchanged to Dulbecco's PBS by ultrafiltration (YM-10, Amicon). The purified M161Ag gave a 43-kDa singlet on SDS-PAGE/silver staining, and its concentration was 60 ng/ml as determined by an ELISA established in our laboratory.[2]

Amino Acid Analysis of M161Ag-- The principle of amino acid analysis used in this study was based on the method of Ishida *et al.* (13). The purified M161Ag was hydrolyzed in 6 M HCl at 110 °C for 24 h in an evacuated sealed tube. To examine Trp content of M161Ag, the sample was also hydrolyzed in 3 M mercaptoethanesulfonic acid at 115 °C for 24 h under evacuated conditions. These hydrolysates were applied to an L8500 amino acid analyzer equipped with an L1050 fluorescence detector (Hitachi, Ltd., Japan) to quantify amino acid derivatives.

Biosynthetic Labeling and Immunoprecipitation-- P39(+) cells (5×10^6) were labeled with 300 µCi of [9,10-^{3}H]palmitic acid in 5 ml of RPMI supplemented with 10% FCS or 10 µCi of [^{14}C]tryptophan in 2.5 ml of Trp-depleted RPMI supplemented with 10% FCS for 24 h at 37 °C. Cells were lysed in lysis buffer (PBS, pH 7.4, 1% Nonidet P-40, 10 mM EDTA, 25 mM iodoacetamide, 2 mM PMSF) for 30 min at room temperature. Lysates were clarified by centrifugation at 10,000 × *g* for 15 min at 4 °C. The supernatants were precleared with protein G-Sepharose (Amersham Pharmacia Biotech). M161Ag was immunoprecipitated with a mAb against M161Ag (MK53), followed by protein G-Sepharose. Nonimmune mouse IgG was used as a control antibody. Immunoprecipitates were washed and analyzed by SDS-PAGE on 10% gel (3). The gels were fixed, soaked in Amplify™ (Amersham) for 30 min, dried, and exposed to Fluorograph film for 10 days (^{3}H) or 12 days (^{14}C).

For ^{75}Se labeling, 10 µCi of [^{75}Se]selenite (Research Reactor Center, University of Missouri, Columbia) was added to P39(+) and P39(−) cells (1 × 10^7) in 10 ml of RPMI supplemented with 10% FCS and cultured for 40 h. Cell lysates were resolved on SDS-PAGE under nonreducing conditions and analyzed with BAS2000 (Fujifilm Co. Tokyo, Japan).

Determination of Cytokines-- THP-1 cells or monocytes from individual healthy donors (1 × 10^6 cells/ml) were stimulated with LPS (5 µg/ml for THP-1 cells, 10 ng/ml for monocytes; *Escherichia coli*, 026:B6, Sigma) and soluble M161Ag (2.4 ng/ml, 6 ng/ml, 12 ng/ml). After 24 h of stimulation, supernatants were removed and cells were lysed by two cycles of freezing/thawing. Cytokine titers in both supernatants and cell lysates were determined by ELISA. The IL-1β ELISA is highly specific for mature IL-1β, and the IL-12 ELISA is highly specific for total human IL-12 (p70 and p40). The cell lines used in this study were free of *Mycoplasma* infection.

RESULTS

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Southern Analysis-- To clarify the origin of M161Ag, we performed Southern analysis using DNAs extracted from human PBMCs, spleen, primary cultures of fibroblasts, and various human cell lines. Hybridizing bands against M161Ag probe were observed in lanes with M161Ag-positive cell lines (3), for example WI38 DNA (Fig. 1*A*), but not in lanes with primary cultures of human fibroblasts (Fig. 1*A*), PBMC, and spleen DNAs (not shown). Fig. 1*B* shows a Southern blot with *Hae*III digests of DNAs from a variety of M161Ag-positive and -negative cell lines. DNA bands appeared in parallel with M161Ag protein expression in the cell lines tested. The size of the *Hae*III digests was variable; ~3 kb in Jurkat(+), CEM(+), and WI38, 7.7 kb in P39(+), and 7 kb in K562(+).

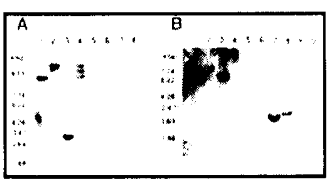

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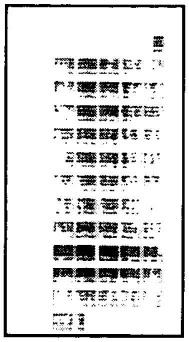
Fig. 1. Southern blotting analysis of the M161Ag gene. *Panel A*, genomic DNAs (10 µg/lane) from WI38 (*lanes 1-4*) and primary cultures of human fibroblasts (*lanes 5-8*) were digested by *Eco*RV (*lanes 1* and *5*), *Kpn*I (*lanes 2* and *6*), *Hae*III (*lanes 3* and *7*), and *Bam*HI (*lanes 4* and *8*). After agarose gel electrophoresis and transfer of the DNAs onto the membrane, the membrane was hybridized with a ^{32}P-labeled M161Ag cDNA. DNA markers are shown alongside. Our interpretation of the three-band profile produced by *Bam*HI is discussed in the text. *Panel B*, genomic DNAs (10 µg/lane) from M161Ag-positive cell lines (*lane 1*, P39(+); *lane 3*, K562(+); *lane 5*, Jurkat(+); *lane 7*, CEM; *lane 8*, WI38) and negative cell lines/cells (*lane 2*, P39(−); *lane 4*, U937; *lane 6*, Jurkat (−); *lane 9*, primarily cultured fibroblast; *lane 10*, PBMC) were digested with *Hae*III, and Southern blotting was performed as in *panel A*.

Cloning of Genomic M161Ag Gene-- Because the genome of M161Ag was absent in normal organs and was not uniform in the M161Ag-positive cell lines, it is unlikely to reside definitely in the human

chromosomal DNA. Thus, genomic analysis was carried out. Finally, one positive clone (CL1) was obtained, which consisted of a single exon based on the sequence on both strands. CL1 was 3141 bp including the 5' regulatory and coding regions of M161Ag, and had 99% identity with 1621 bp of M161Ag cDNA obtained from the P39(+) cDNA library (Fig. 2A). One base (C (cDNA) to T (CL1)) transition at CL1 1820 bp caused a His to Tyr conversion, and a three-nucleotide (corresponding to Ala) in-frame insertion at CL1 2261 bp resulted in generation of a putative 429-amino acid precursor protein.



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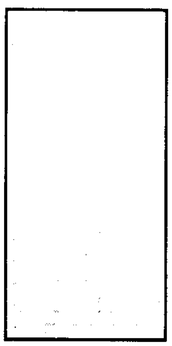

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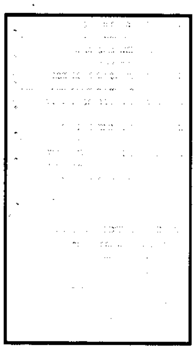

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Fig. 2. Nucleotide sequence analysis of the M161Ag gene. *Panel A*, alignment of nucleotide sequences of the M161Ag cDNA clone from P39(+) cells (p161-11), the M161Ag gene from WI38 cells (CL1), and the *M. fermentans* genomic clone, 5MF (encoding P48). Identical nucleotides in these sequences are *boxed*, and the consensus sequence is shown underneath. One nucleotide was substituted (C to T) at position 1820 bp, and three nucleotides (GCA) were inserted at position 2261-2263 bp in CL1. The poly(A) sequence appeared 25 bp downstream of AATAAA corresponding to 3000-3006 bases of CL1. M161Ag gene (from 1343 bp to 1964 bp) had 99% identity with 621 bp of 5MF. One nucleotide (T) at position 1748 in CL1 was deleted in 5MF. Consensus sequences for −35 and −10 promoter regions, the Shine-Dalgarno (SD) sequence, the ATG translation starting codon, and the TAA stop codon are underlined. These sequence data will appear in the DDBJ, EMBL, and GenBank™ under accession number AB008823. *Panel B*, alignment of the deduced amino acid sequence of M161Ag and P48. The N-terminal 114 amino acids were highly homologous in the two proteins. Common amino acids are shown by *asterisks*.

Unexpectedly, the M161Ag genomic clone contained the Pribnow box at the −35 and −10 promoter portions and the Shine-Dalgarno ribosomal binding site ~10 bp upstream of the translation start codon (Fig. 2*A*), both suggestive of a prokaryote gene. Additionally, the eukaryote-like polyadenylation signal AATAAA and the subsequent poly(A) sequence observed in M161Ag cDNA mostly resided in the genome. Hence, we conclude that the M161Ag gene was derived from a prokaryote.

The M161Ag gene was 99% identical to a *M. fermentans* gene 5MF (621 bp) encoding a monocytic differentiation/activation factor of P48 (Fig. 2*A*) (4), which was released in the data base very recently (3). However, at the amino acid sequence level, M161Ag was only 30% identical to P48; M161Ag and P48 shared most of the N-terminal 114 amino acids including a 24-amino acid signal peptide but were diverged into two distinct proteins by their respective C-terminal 315 and 71 amino acids, which were totally unrelated (Fig. 2*B*). This reflected one nucleotide (T) insertion at CL1 1748 bp and cDNA 354 bp, resulting in a frameshift allowing for translation of the subsequent 315 amino acids of M161Ag. This frameshift also yielded 5 TGA codons. Thus, the two gene products differed in primary structure and predicted molecular mass; the M161Ag gene from WI38 cells encoded a 429-amino acid precursor protein with the molecular mass of 47,905 Da, whereas the P48 gene encoded a 185-amino acid protein of 20,406 Da.

We next analyzed RT-PCR products from various M161Ag-positive cell lines to resolve the discrepancies between the cDNA and genomic clones at positions CL1 1820 and 2261-3. Table I summarizes the three isoforms of M161Ag; M161Ag-1 has His^{139} without Ala^{285} insertion, M161Ag-2 has Tyr^{139} and no Ala^{285}, and M161Ag-3 is a Tyr^{139}/Ala^{285} form. Their distributions in our cell lines are shown in Table I. M161Ag isoforms must possess C3-activating function because all of these cell lines induced homologous C3 deposition on their surface (data not shown).

Table I
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M161Ag Is a Gene Product of M. fermentans-- Two methods were used to determine whether M161Ag is related to a *M. fermentans* gene product. Firstly, *Mycoplasma* infection was confirmed by RT-PCR using primers of *Mycoplasma* genus-specific rRNA. There was a correlation in the results of PCR analysis between M161Ag expression and *Mycoplasma* infection (Fig. 3).

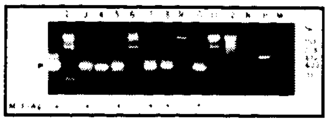

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Fig. 3. PCR analysis of *Mycoplasma* infection. *Mycoplasma* genus-specific rRNA was amplified in DNAs from M161Ag-positive and -negative cell lines/cells. M161Ag (detected by flow cytometry) and the PCR band were observed in parallel. U937 cells used in this experiment did not activate homologous complement and gave a PCR band with slightly faster mobility (*lane 4*) but was exceptionally M161Ag-negative, suggesting that this cell line was infected with another *Mycoplasma* species. *Lane 1*, P39(+); *lane 2*, P39(−); *lane 3*, K562(+); *lane 4*, U937; *lane 5*, Jurkat(+); *lane 6*, Jurkat(−); *lane 7*, CEM; *lane 8*, WI38; *lane 9*, primary culturs of human fibroblasts; *lane 10*, HT1008; *lane 11*, PBMCs; *lane 12*, spleen. *N*, deionized distilled water; *P*, positive control. DNA marker sizes are indicated at the *right margin*. The *arrowhead* indicates the DNA from *Mycoplasma* genus-specific rRNA.

M161Ag cDNA contained 5 TGA codons which were initially presumed to be translated into selenocysteines in human cells (14). Because the TGA codon is translated into Trp in *Mycoplasma* species (15), we next performed amino acid analysis of the M161Ag purified from P39(+) cells. The Trp peak was detected in the elution profiles of purified M161Ag samples (data not shown). Biosynthetic labeling of P39(+) cells with [^{14}C]Trp, and immunoprecipitation resulted in a labeled 43-kDa band aligned with M161Ag (Fig. 4*A*). Virtually no selenocysteine could be identified in the cell lines shown in Fig. 1 by the ^{75}Se incorporation test, under which cellular glutathione peroxidase (having one selenocysteine) (16) was visualized (Fig. 4*B*). Thus, M161Ag is a gene product of *M. fermentans*. Host genomic integration, however, could not be ruled out yet.

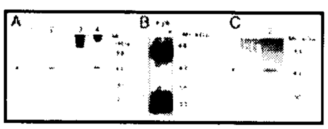

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Fig. 4. Biosynthetic labeling of P39(+) cells. *A*, M161Ag contains Trp residues. P39(+) cells were biosynthetically labeled with [^{14}C]Trp for 24 h at 37 °C, and the protein was immunoprecipitated with nonimmune mouse IgG (*lanes 1* and *3*) or mAb against M161Ag (*lanes 2* and *4*). The precipitates were analyzed by SDS-PAGE followed by fluorography (*lanes 1* and *2*) or immunoblotting using M161 mAb (*lanes 3* and *4*). The *arrowhead* indicates M161Ag. The high molecular weight material seen in the top of the gel reflects the Ab used for immunoprecipitation (*lanes 3* and *4*). *B*, ^{75}Se was not incorporated into M161Ag. P39(−) and P39(+) cells were labeled with [^{75}Se]selenite for 40 h at 37 °C. The cell lysates were analyzed by SDS-PAGE/autoradiography. ^{75}Se was incorporated into the 50-60-kDa molecules (unidentified) and cellular glutathione peroxidase (*open arrowhead*) but not into M161Ag. *C*, M161Ag is palmitoylated. P39(+) cells were labeled with [^{3}H]palmitate, and the protein was immunoprecipitated with nonimmune mouse IgG (*lane 1*) or MK53 (*lane 2*). The precipitates were analyzed by SDS-PAGE and fluorography.

M161Ag Is Modified by Palmitate-- Prokaryotic signal peptidase II cleaves precursor polypeptides upstream of a Cys residue to which a lipid moiety is then attached (17). M161Ag carried in its N terminus a four-amino acid motif (AVSC) characteristic of bacterial lipoproteins. To test the lipid modification, P39(+) cells were biosynthetically labeled with [9,10-^{3}H]palmitic acid. As shown in Fig. 4C, palmitic acid was incorporated into M161Ag. Thus, M161Ag is a lipoprotein.

Inflammatory Cytokine-inducing Activity of M161Ag-- M161Ag shared the identical N-terminal 114 amino acids with P48, a cytokine (IL-1, TNF-α, and IL-6) inducer. This prompted us to test whether these cytokines were produced in monocytes and THP-1 cells in response to M161Ag stimulation. As shown in Fig. 5A, purified soluble M161Ag induced IL-1β production, but not secretion, in THP-1 cells. Minimal TNF-α and IL-6 were detected in conditioned media. These cytokines were produced in an M161Ag dose-dependent manner.

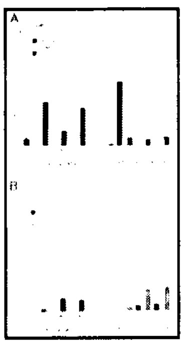

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Fig. 5. Induction of cytokine production by M161Ag. *A*, induction of IL-1β in THP-1 cells by M161Ag. THP-1 cells (1×10^6/ml) were cultured in 24-well dishes for 24 h in the presence of LPS (5 µg/ml) or M161Ag (6 ng/ml, 12 ng/ml). Cytokines were quantified in both supernatants and cell lysates by an ELISA specific for human IL-1β (*closed column*), TNF-α (*open column*), and IL-6 (*shadowed column*). Determinations were performed in triplicate, and results are expressed as means ± S.D. Values represent the total amounts of cytokines produced by 10^6 cells. Results are representative of two separate experiments. *B*, cytokine secretion in human monocytes induced by M161Ag. Monocytes were stimulated with LPS (10 ng/ml) or M161Ag (2.4 ng/ml, 12 ng/ml) for 24 h, and cytokines were quantified as described above. Determinations were performed in triplicate and are expressed as means ± S.D. Results are representative of three separate experiments from different healthy donors. The viabilities of THP-1 cells and monocytes were not affected by stimulation with LPS or M161Ag.

M161Ag was more potent as a cytokine inducer on human monocytes than THP-1 cells (Fig. 5*B*). Secretion of IL-1β, TNF-α, and IL-6 from monocytes was enhanced more effectively by M161Ag than by LPS. Moreover, M161Ag led to the secretion of IL-10 and IL-12 from human monocytes (Fig. 6). These cytokines play crucial roles in both innate and acquired immune responses. Other cytokines, IL-2 and IL-4, could not be detected in the same system (data not shown).

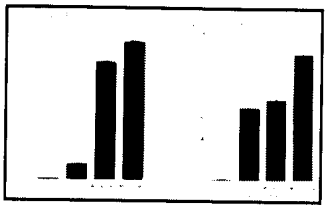

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Fig. 6. M161Ag stimulates monocytes to secrete IL-10 and IL-12. Human monocytes were stimulated with LPS (10 ng/ml) or M161Ag (2.4 ng/ml, 12 ng/ml). After 24 h, the supernatants were harvested, and IL-10 and total IL-12 (p70 and p40) levels were determined by ELISA. Unstimulated cells were cultured in parallel without stimulators. Determinations were performed in triplicate, and results are expressed as means ± S.D. Results are representative of two separate experiments.

DISCUSSION

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We initially expected M161Ag to be a human gene product, because its cDNA had a polyadenylation signal followed by the poly(A) tail and the protein was originally detected in bone marrow cells of patients with leukemia undergoing chemotherapy and in those with aplastic anemia.[3] Additionally, the 5 TGA codons in the ORF of this protein could be read into selenocysteines if it were a human protein. At that time, no protein or nucleotide sequence similar to M161Ag was found in the data base.

The findings of the present study can be summarized as follows. 1) 5'-UT of cDNA and genomic sequences predicted the presence of the prokaryote promoter and ribosomal binding site (Fig. 2). 2) A poly(A) tail-like sequence as well as an AATAAA polyadenylation signal was conserved not only in the cDNA but also in the genome (Fig. 2). 3) There was a correlation between M161Ag expression and *Mycoplasma* infection (Fig. 3). 4) Homology searches indicated that the P48 monocyte differentiation/activation factor gene originating from *M. fermentans* (accession number U70254) was highly homologous to our M161Ag cDNA and genome. 5) The TGA codons encoded Trp but not selenocysteine residues in the purified protein as in *Mycoplasma* proteins. Therefore, we conclude that M161Ag is a protein of *Mycoplasma* but not human origin. However, the size variety of genomic Southern analysis remains unexplained. Strain-to-strain difference and/or genomic integration of the M161Ag gene may account for this unusual result.

These points are reminiscent of the suggestion that mitochondria are derived from parasitic bacteria because they have their own genome and proteins. Some of the genomic structure still bear the marks of prokaryotic origin. An intriguing point is that the M161Ag mRNA partly mimics those of eukaryotes, and this may be advantageous for attainment of stable dynamics and steady-state level in the parasitic environment (18).

M161Ag is a multifunctional protein with abilities of C activation and cytokine induction. P48 of *M. fermentans* induces the production of IL-1B, TNF-α, and IL-6 in human monocytes and monocytic cell lines (4-8). Because the amino acid sequences were largely different throughout the C-terminal regions between M161Ag and P48, the IL-1β-, TNF-α-, and IL-6-inducing activity must be mapped within the N-terminal domain conserved between these two proteins. However, M161Ag, but not P48,

stimulates monocytes to induce IL-10 and IL-12 which affect the polarization and development of naive T-helper cells. Again, M161Ag has C-activating ability which has not been determined in P48. The stretched sequence of M161Ag including 5 Trp residues may play a role in the latter functions.

M161Ag is a putative membrane protein with a lipid anchor since M161Ag was palmitoylated (Fig. 4C). The lipid moiety on bacterial lipoproteins strongly potentiates the humoral as well as the cellular immune responses (19). Indeed, MALP-2 (a recently isolated *M. fermentans*-derived 2-kDa lipopeptide, macrophage-activating lipopeptide-2), carries 1 mol of C16:0 and an additional mole of a mixture of C18:0 and C18:1 fatty acids per lipopeptide molecule (20) and acts as an inducer of NO at picomolar concentrations (20). Surprisingly, the amino acid sequence of this lipopeptide was entirely consistent with the N-terminal 14-amino acid sequences of M161Ag and P48. It is likely that soluble forms of this *M. fermentans* gene product confer another function on macrophages besides C-activation and cytokine production. However, the mechanisms whereby soluble M161Ag is generated from the membrane-associated forms to express its functions still remain unknown.

M. fermentans is a mycoplasma species capable of infecting humans and has been suspected of serving as a cofactor of AIDS development (21, 22). Several groups (10, 12) speculated that *M. fermentans* facilitates depletion of T cells or immature myelomonocytic cells, favoring the progression of functional immunodeficiency in AIDS. Yet, the products of *M. fermentans* responsible for immune modulation, polyclonal B or T cell activation, cytokine production, and cytocidal effect (23-26) have not been identified. A possible interpretation is that M161Ag and/or P48 is a molecule relevant to AIDS progression. C3-activating function of M161Ag is also consistent with the observation that C3 deposition is induced on $CD4^+$ T cells of HIV-infected individuals (27, 28). Coinfection of *M. fermentans* with HIV may actually support progression to AIDS in latent patients via the functions of M161Ag.

These results also explain why M161Ag-positive myeloid cell lines were obtained after most of the cells died. Like human myeloid cell lines P39(+) and K562(+), infection with *M. fermentans* may cause cell death in affected cells, and some that survive are persistently infected with *M. fermentans* and are M161Ag-positive. The parasitic growth of *M. fermentans* may be regulated by signals related to cell death, since M161Ag synthesis is induced by x-irradiation and Fas stimulation and up-regulated with TNF-α (3).

Our sequential studies showed that the *M. fermentans* gene product M161Ag had dual functions: complement activation and cytokine induction. Once M161Ag is expressed because of latent infection of *fermentans*, it converts self cells to non-self and elicits innate immune responses via activation of C3/C5 and monocytes. However, the roles of autologous C3 activation and deposition on host cells and Th1-activating cytokine production in the acquired immune responses are still poorly understood. Furthermore, parasitic infection of *M. fermentans* has been associated with oncogenic properties (29, 30). These issues will be further clarified using recombinant M161Ag and deletion mutants, and such studies are currently in progress in our laboratory.

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FOOTNOTES

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[1] The abbreviations used are: C, complement; CM, conditioned media; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear cell; PMSF, phenylmethylsulfonyl fluoride; IL, interleukin; TNF, tumor necrosis factor; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s); bp, base pair(s); HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

[2] S. Kikkawa, M. Matsumoto, M. Kurita, M. Nishiguchi, and T. Seya, manuscript in preparation.

[3] M. Matsumoto and T. Seya, unpublished data.

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